SUPPLEMENTARY MATERIAL

DETERMINANTS OF THE PRO-INFLAMMATORY ACTION OF AMBIENT PARTICULATE MATTER IN IMMORTALISED MURINE MACROPHAGES

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CONTENTS

METHODS

A full description of the methods summarised in this supplementary section are available at: http://www.rivm.nl/bibliotheek/rapporten/863001002.pdf. Further descriptions of the sampling campaign and compositional analysis have been presented previously (Gerlofs-Nijland et al, 2007; Janssen et al, 2008).

Chemicals and cell lines: RPMI 1640 medium without glutamine was obtained from HyClone (Cramlington, UK). Glutamax I supplement-containing L-alanyl-glutamine was from Life Technologies (San Giuliano Milanese, Italy). LPS (serotype 026:B6) was obtained from Sigma (Milan, Italy). [^{3}H]AA (60-100 Ci/mmol) was from DuPont Nuclear (New England). Fetal Bovine Serum endotoxin <10 EU/ml was obtained from Hyclone (Cramlington, UK). Diethylenetriaminepentaacetic acid (DTPA) was obtained from Sigma (Poole, UK) and recombinant endotoxin neutralizing protein (rENP) from Cape Cod Incorporated (Falmouth, MA, USA). The mouse monocyte/macrophage cell line RAW 264.7 was kindly donated by Prof. B. Brune, University of Erlangen-Nurnberg, Germany and maintained at the Istituto Superiore di Sanita as previously described (Guastadisegni et al. 1997).

Particle collection and extraction: A High Volume Cascade Impactor with a multi-stage round slit nozzle impactor was used to collect PM fractions onto polyurethane foams (PUF) (Kavouras and Koutrakis, 2001; Demokritou et al., 2002). Prior to use the PUF filters were cleaned by sonicatation for between 30-60 minutes in 100% methanol and air dried, after which their weight was determined using an analytical balance. After sampling the foams were conditioned and re-weighed. A detailed description of the procedure has been published previously (Cassee et al. 2003). The

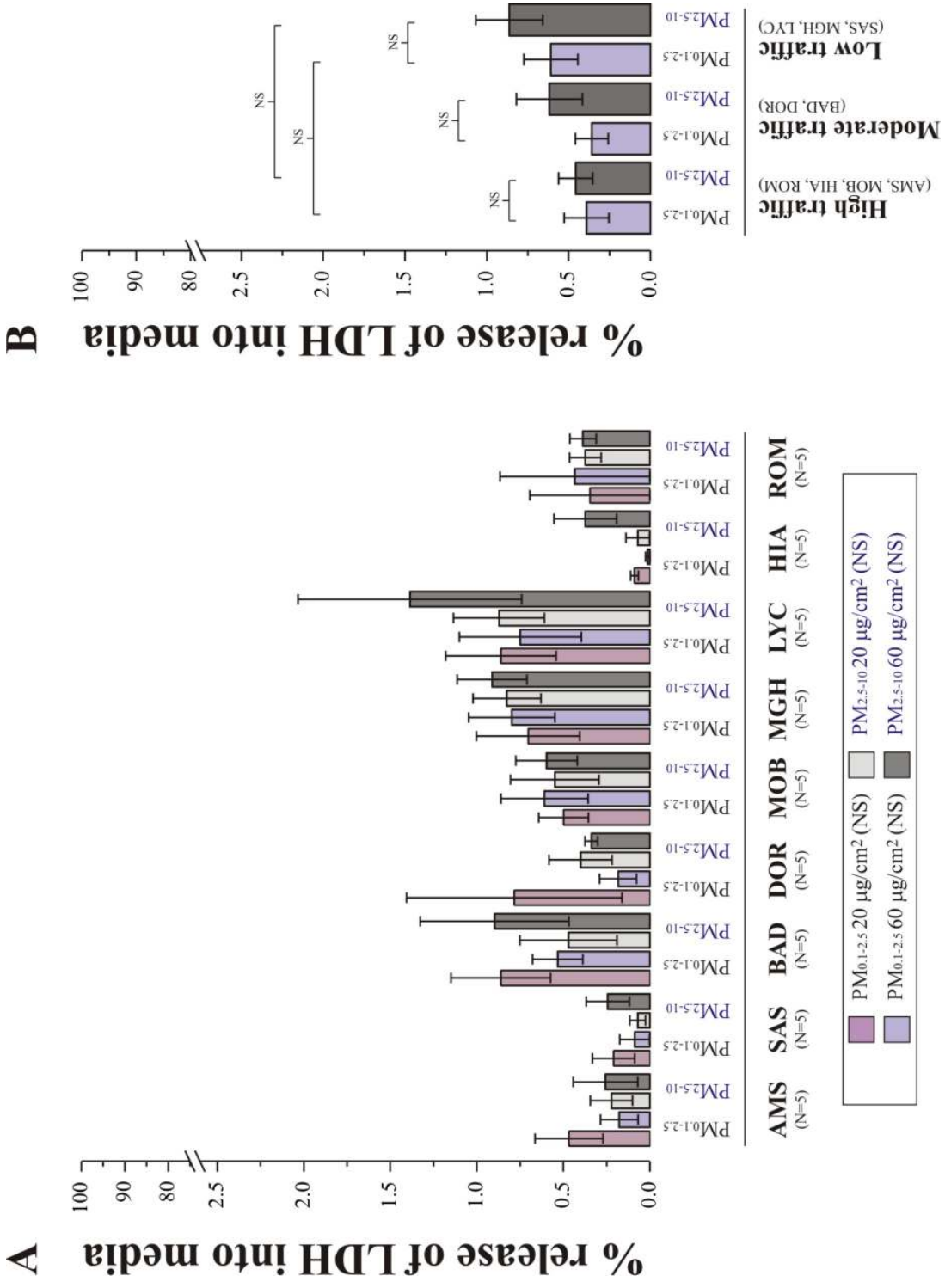
PM was extracted from the foams according to the method of Salonen et al (2000). Briefly, 20 ml methanol was added to 50 ml falcon tubes containing the foams and the tubes vortexed violently before being placed in an ultrasonic bath for 30 minutes. After this treatment the supernatant was decanted into a round-bottom flask and the procedure repeated. The final pooled methanol extract was subsequently concentrated using a rotary evaporator at 30 ºC, low pressure, for approximately 15 minutes, until only 1 ml of suspension remained. This suspension was subsequently sonicated in the flask for several seconds to remove the PM adhered to the wall of the flask and divided into pre-weighed Eppendorf-tubes. The Eppendorf tubes were then transferred to an oven at 30 °C overnight and afterwards conditioned for 24 hrs. The amount of PM per Eppendorf tube was subsequently determined using an analytical balance. The tubes were stored at –20 ºC until analysis for their composition and -80^{o}C for their inflammatory potential.

Chemical characterization: The collected PM was characterized for an extended suite of elements, ions and polycyclic aromatic hydrocarbons (PAHs). PM for elemental composition analysis was resuspended in 0.5 ml of water and transferred into a digestion vial. The sample tube was subsequently rinsed with 1 ml water and 3 times with 1 ml of a pre-prepared dilute aqua regia solution: 1 ml 65% HNO_3 + 3.3 ml 37% HCl + 3 ml water) and the rinse solutions added to the digestion vial, with the final volume made up to 10 ml. Following microwave digestion an additional 5 mls of water was added to the sample, which was then transferred to autosampler vials for analysis by inductively coupled plasma mass spectrometry (ICPMS) for the following elements: Li, Be, Br, Na, Mg, Si,Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni,

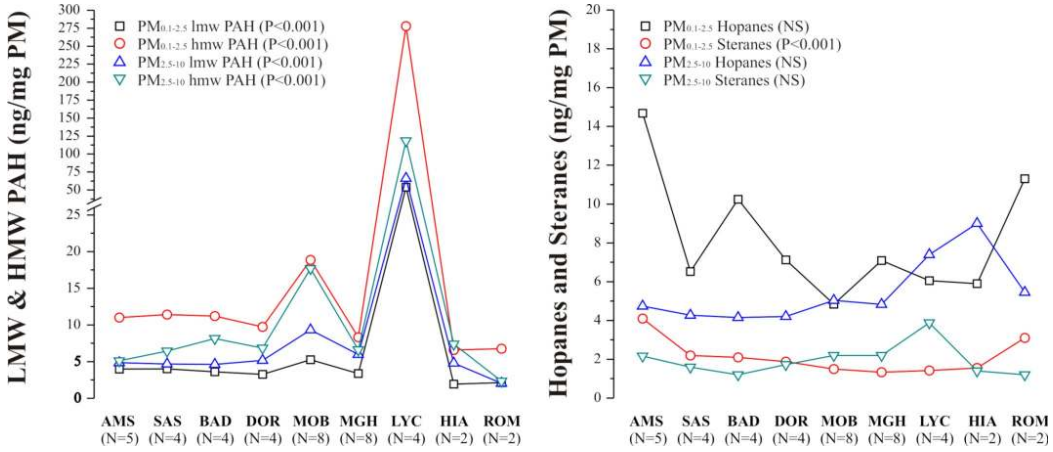
Cu, Zn, As, Se, Sr, Mo, Cd, Sb, Ba, La, Ce, Nd, Hf, Hg, Sm, Tl, Pb, and U. Rh was employed as an internal standard.

Ionic species: PM suspensions (1 mg) in methanol were evaporated to dryness and dissolved in water by sonication. Following filtration to remove any insoluble material the samples were analyzed using ion-chromatography for chloride (Cl-), nitrate (NO_3^-) and sulphate (SO_4^{2-}). These anions were analysed using a Dionex guard column (AG-4A), separation column (Dionex AS-4A) and pulsed electrochemical detector (Dionex-PED). Ammonium (NH^{4+}) was initially chlorinated and then derivatised into the blue complex of 5-aminosalicylate. The absorption of this complex was then measured at 660 nm using a continuous flow analyzer system (CFA) and quantified against appropriate standards.

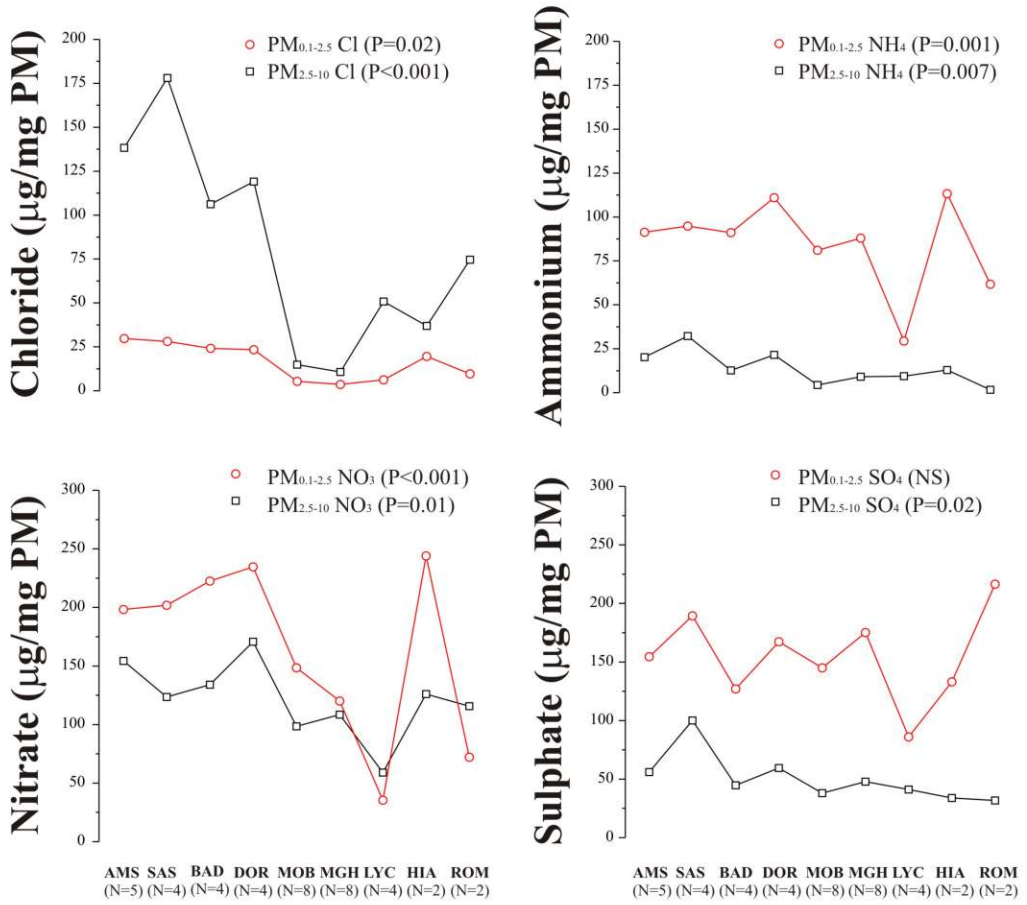
Polycyclic aromatic hydrocarbons (PAH): The supplied PM suspension (6 mg) in methanol was evaporated to dryness prior to the addition of a 50 µl aliquot of the of internal standards (6 deuterated PAHs) and 50 ml dichloromethane/isohexane (1:1). The sample was then sonicated and filtered prior to being concentrated by evaporation to near dryness and mixed with 0.5 ml of a standard solution of 2.4-dichlorobenzyltetradecylether, which was used to correct for the variation of the injection volume. 1 µl of this extract was then injected (splitless mode) at 290 ^oC onto a 30 m 0.25 mm WCOT DB-5MS column (film 0.25 µm) using a column temperature programmed from 90 – (ramp rate) - 160– (ramp rate) – 290 ^oC in a Fisons 8000 series gaschromatograph equipped with an Interscience MD800 mass-spectrometer with EI in SIR mode.


A
% release of LDH into media
100
90
80
2.5
2.0
1.5
1.0
0.5
0.0
PM0.1-2.5
PM2.5-10
AMS (N=5)
SAS (N=5)
BAD (N=5)
DOR (N=5)
MOB (N=5)
MGH (N=5)
LYC (N=5)
HIA (N=5)
ROM (N=5)
PM0.1-2.5 20 µg/cm² (NS)
PM0.1-2.5 60 µg/cm² (NS)
PM2.5-10 20 µg/cm² (NS)
PM2.5-10 60 µg/cm² (NS)
B
% release of LDH into media
High traffic
(AMS, MOB, HIA, ROM)
Moderate traffic
(BAD, DOR)
Low traffic
(SAS, MGH, LYC)
NS

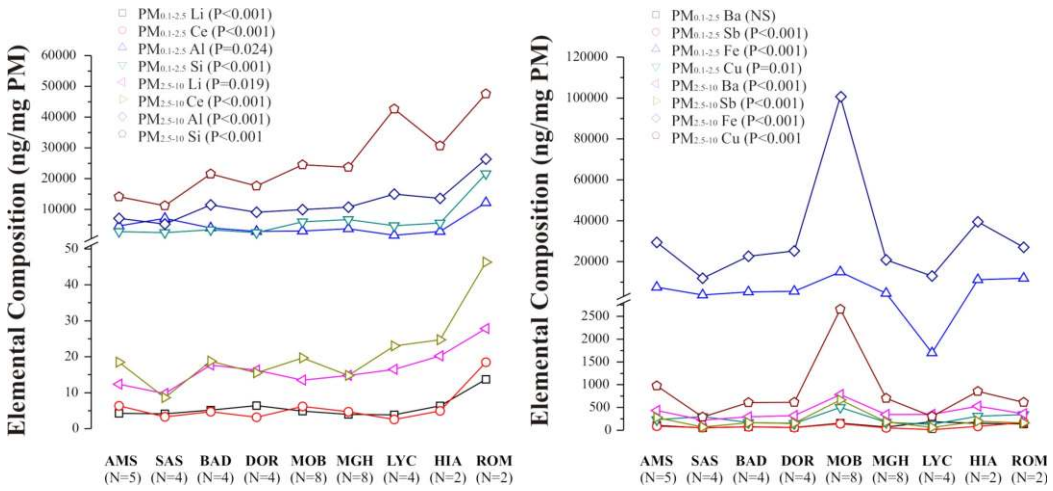
Supplemental Figure 1: Percentage of intracellular LDH released into cell culture media following a 5 hour incubation with coarse and fine PM at 20 and 60 μg/cm^2. Results are expressed as LDH activity in the culture supernatant, minus that observed in particle free control cells, expressed as a percentage of total LDH activity in the cell lysate. Data are presented for 9 different sites throughout Europe (panel A), and by traffic classification: high (n=17), moderate (n=8) and low (n=16) (panel B). In panel A bars represent the site means ± SEM of n=2-8 PM sample incubations, with each experiment being performed on two independent occasions in duplicate. Abbreviations for site identification are as specified in materials and methods section of the main paper.



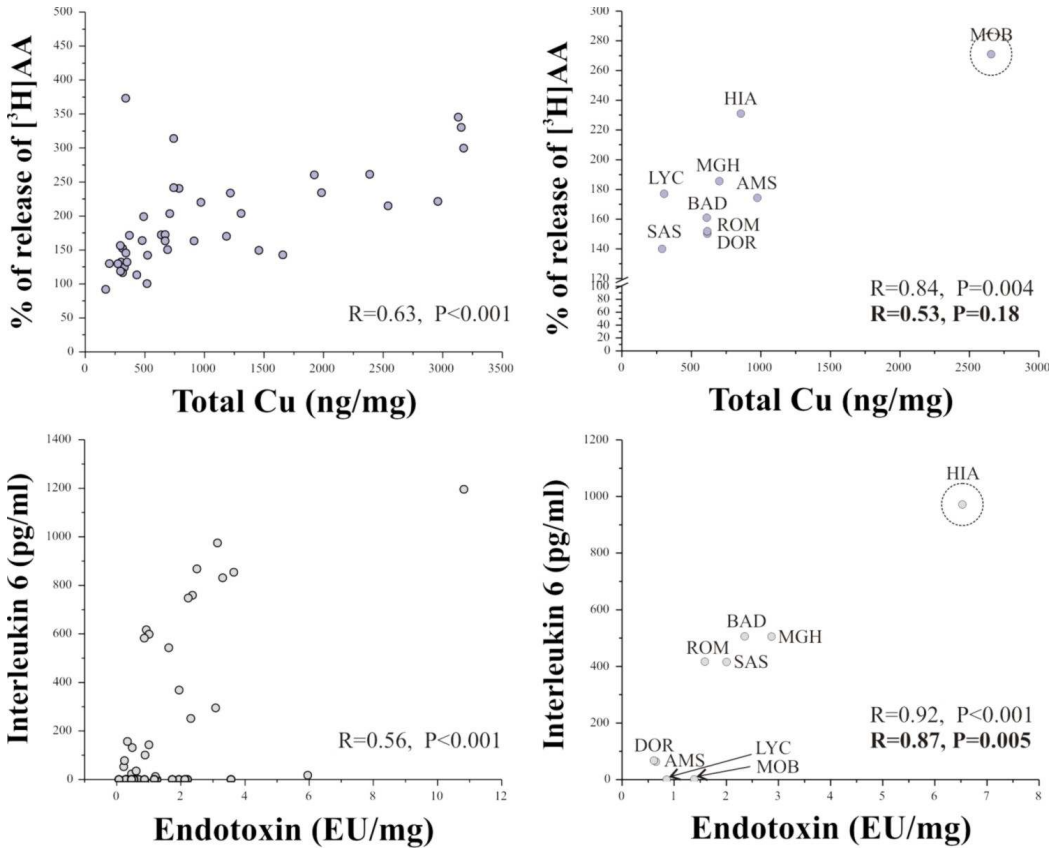
Supplemental figure 2: Mean site concentrations of high and low molecular weight PAHs (left hand panel) and hopanes/steranes (right hand panel) associated with $PM_{0.1-2.5}$ and $PM_{2.5-10}$ at the 9 selected sites in the current study. The results of a One Way ANOVA indicating contrasts in the site means over the sampling periods are presented in the inset.



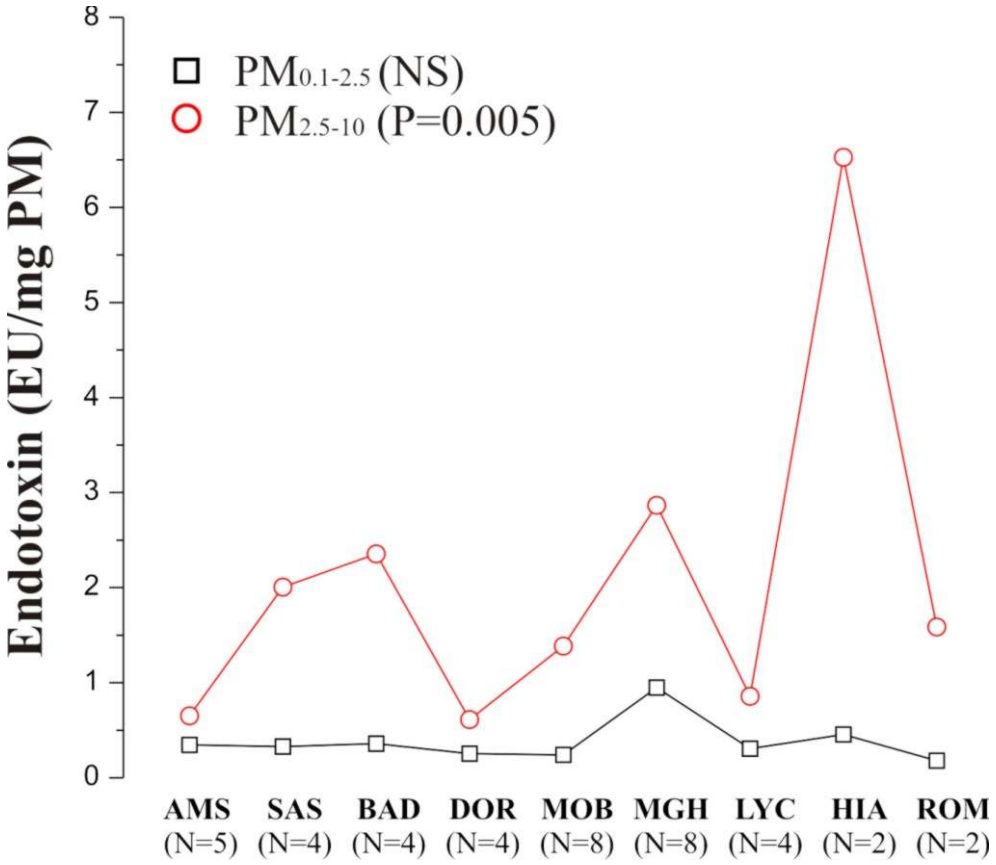
Supplemental figure 3: Mean site concentrations of selected ionic species in $PM_{0.1\text{-}2.5}$ and $PM_{2.5\text{-}10}$ collected from the 9 selected sites in the current study. The results of a One Way ANOVA indicating contrasts in the site means over the sampling periods are presented in the inset.



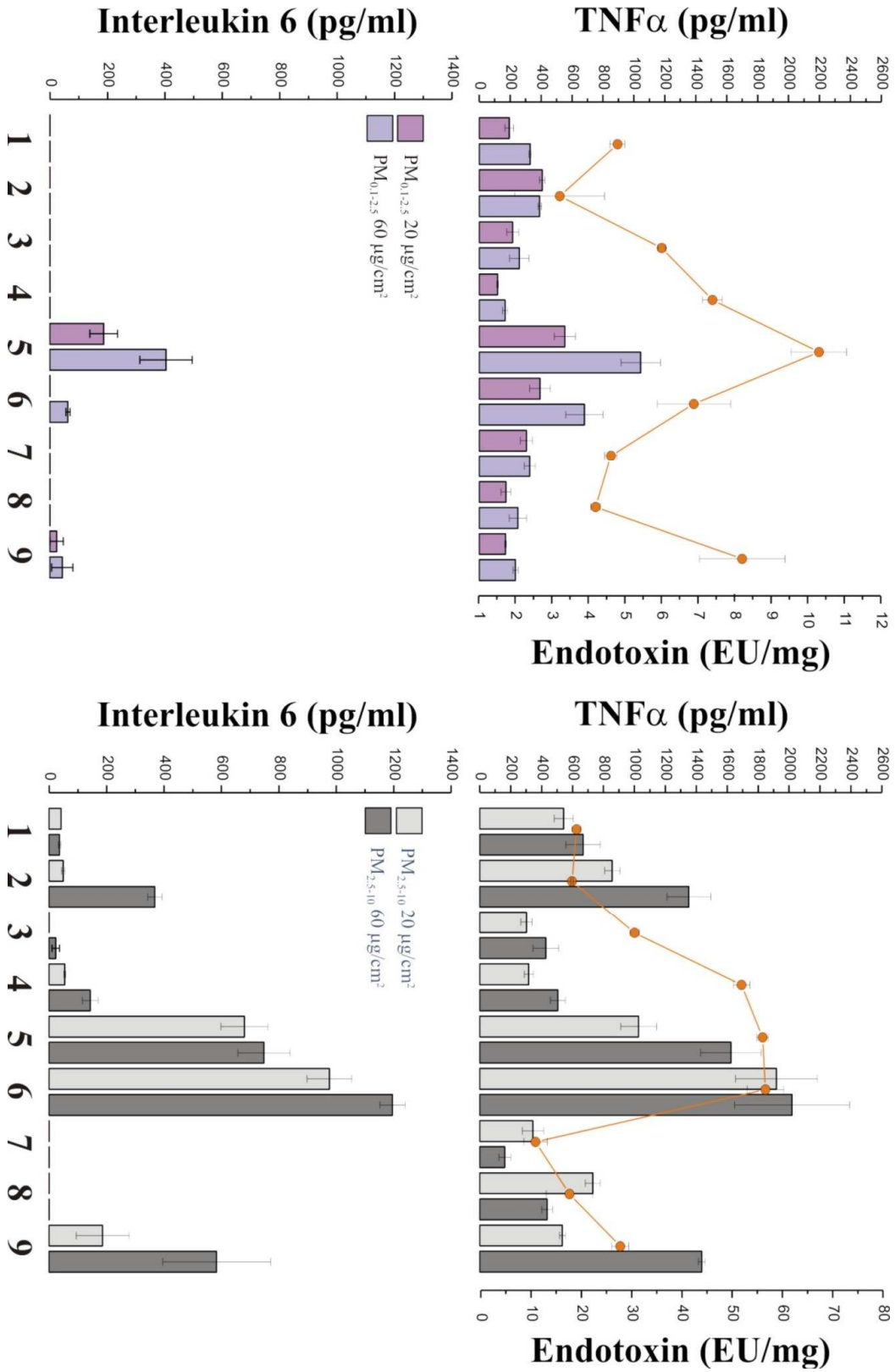
Supplemental figure 4: Mean site concentrations of selected elements associated with $PM_{0.1\text{-}2.5}$ and $PM_{2.5\text{-}10}$ at the 9 selected sites in the current study. The elements in the left hand panel reflect crustal contributions to the PM, whereas those in the right hand panel are reflective of vehicle abrasion processes, particularly tire wear. The results of a One Way ANOVA indicating contrasts in the site means over the sampling periods are presented in the inset.



Supplemental figure 5: Correlations between $PM_{2.5-10}$ total Cu (upper panels) and endotoxin contents (lower panels) with the releases of [^{3}H]AA and IL-6. Figures in the right hand panels illustrate the associations based on the individual site means, with the inset results of the correlation analysis performed with and without (bold text) the inclusion of the values associated with MOB and HIA for the AA and IL-6 responses respectively.



Supplemental figure 6: Mean site concentrations of endotoxin in $PM_{0.1\text{-}2.5}$ and $PM_{2.5\text{-}10}$ collected from the 9 selected sites in the current study. The results of a One Way ANOVA indicating contrasts in the site means over the sampling periods are presented in the inset.


TNFα (pg/ml)
Endotoxin (EU/mg)
Interleukin 6 (pg/ml)
PM0.1-2.5 20 μg/cm²
PM0.1-2.5 60 μg/cm²
TNFα (pg/ml)
Endotoxin (EU/mg)
Interleukin 6 (pg/ml)
PM2.5-10 20 μg/cm²
PM2.5-10 60 μg/cm²
1 2 3 4 5 6 7 8 9

Supplemental figure 7: Comparison of the concentration of IL-6 and TNFα released following a 5 hour incubation (20 and 60μg/cm^2) with 9 test PM samples ($PM_{0.1-2.5}$ and $PM_{2.5-10}$) collected as part of the HEPMEAP project. Data are presented as mean ± SEM of three independent experiments performed in triplicate. The concentration of endotoxin associated with each of the samples is illustrated (orange circles). Endotoxin in these samples were analysed using the kinetic Limulus Amebocyte Lysate (LAL) test, BioWhittaker Molecular Applications (Walkersville MD, U.S.A.) which differed from the method employed in the initial pre-screening exercise. Samples are labelled 1-9 and reflect: 1, Dordrecht summer, moderate traffic; 2, Sassenheim, winter, low traffic; 3, Amsterdam, spring, high traffic; 4, Amsterdam, summer, high traffic; 5, the HIA road tunnel, autumn, high traffic; 6, HIA road tunnel, spring, high traffic; 7, Munich Ost Bahnhof, spring, high traffic; 8 Munich Ost Bahnhof, spring, high traffic; and 9, Rome, spring, high traffic. These data illustrate the equivalence of the IL-6 and TNFα response to PM, with more consistent signals associated with the later cytokine. The correlation between the responses in these cytokines and the PM endotoxin content is also illustrated.

Supplemental Table 1*:* The degree of association (Pearson's correlation coefficients) between the particle associated PAHs samples at 9 different sites throughout Europe with the concentrations of AA and IL-6 released or produced by RAW 264.7 cells incubated with 60 µg/cm^2 of coarse (PM $_{10\text{-}2.5}$) and fine (PM $_{2.5\text{-}01}$) PM. Analyses were performed treating each PM sample separately (black text, n=40-41) or restricted to the site means (blue text, n=9).

Fraction	Endpoint		PM Associated Organics	
			lmw PAH[1]	*hmw* PAH[2]
$PM_{2.5\text{-}10}$	**AA**	Correlation coefficent	0.024	0.125
		N	41	41
		Correlation coefficent	0.022	0.041
		N	9	9
$PM_{0.1\text{-}2.5}$	**AA**	Correlation coefficent	0.489***	0.490***
		N	41	41
		Correlation coefficent	0.757*	0.757*
		N	9	9
$PM_{2.5\text{-}10}$	**IL-6**	Correlation coefficent	-0.280	-0.278
		N	40	40
		Correlation coefficent	-0.410	-0.409
		N	9	9
$PM_{0.1\text{-}2.5}$	**IL-6**	Correlation coefficent	-0.160	-0.163
		N	40	40
		Correlation coefficent	-0.246	-0.233
		N	9	9

Statistically significant correlations are shown as follow * p<0.05, **p<0.01, ***p<0.001.

1. Low molecular weight PAHs (<180 g mol^{-1}) reflected the sum of naphthalene, 1-methyl-naphthalene, biphenyl, 2,6-dimethyl-naphthalene, acenaphthylene, acenaphthene, 2,3,5-trimethyl-naphthalene, flourene, phenanthrene and anthracene.
2. High molecular weight PAHs (>180 g mol^{-1}) reflected the sum of 1-methyl-phenanthrene, fluoranthene, pyrene, benz[a]antracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo(e)pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-cd]-pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene.

Supplemental Table 2: The degree of association (Pearson's correlation coefficients) between the PM associated ionic species and the concentrations of AA and IL-6 released or produced by RAW 264.7 cells incubated with 60 µg/cm^2 of coarse (PM $_{10\text{-}2.5}$) and fine (PM $_{2.5\text{-}01}$) PM. Analyses were performed treating each PM samples separately (black text, n=40-41) or restricted to the site means (blue text, n=9)..

Fraction	Endpoint		Ionic Species			
			NH_4	Cl	NO_3	SO_4
$PM_{2.5\text{-}10}$	AA	Correlation coefficent	-0.346*	-0.375*	-0.257	-0.249
		N	41	41	41	41
		Correlation coefficent	-0.479	-0.717*	-0.323	-0.508
		N	9	9	9	9
$PM_{0.1\text{-}2.5}$	AA	Correlation coefficent	-0.276	0.346	-0.170	0.013
		N	41	41	41	41
		Correlation coefficent	-0.365	0.003	-0.179	-0.633
		N	9	9	9	9
$PM_{2.5\text{-}10}$	IL-6	Correlation coefficent	-0.148	-0.106	-0.011	-0.133
		N	40	40	40	40
		Correlation coefficent	-0.011	-0.145	0.108	-0.113
		N	9	9	9	9
$PM_{0.1\text{-}2.5}$	IL-6	Correlation coefficent	0.092	-0.202	-0.197	0.224
		N	40	40	40	40
		Correlation coefficent	0.418	-0.102	0.27	-0.03
		N	9	9	9	9

Statistically significant correlations are shown as follow * $p<0.05$

Supplemental Table 3: The degree of association (Pearson's correlation coefficients) between the elemental components of $PM_{10-2.5}$ and $PM_{2.5-01}$ collected at 9 different sites throughout Europe with their induction of AA and IL-6 release and production from RAW 264.7 cells. Analyses were performed on all samples (black text, n=41) and on the restricted site means (blue text, n=9) to show variation by site.

Fraction	Endpoint		Elements												
			Fe	Al	Si	Zn	Cu	Ba	Sb	Mn	Pb	Ce	Cr	Ni	V
$PM_{2.5-10}$	AA	Correlation coefficent	0.590***	-0.115	0.074	0.391*	0.629***	0.561***	0.601**	0.556***	0.317*	0.010	0.592***	-0.093	-0.355*
		N	41	41	41	41	41	41	41	41	41	41	41	41	41
		Correlation coefficent	0.865**	-0.061	0.101	0.515	0.844**	0.946***	0.810**	0.928***	0.474	0.008	0.832**	-0.105	-0.361
		N	9	9	9	9	9	9	9	9	9	9	9	9	9
$PM_{0.1-2.5}$	AA	Correlation coefficent	-0.068	0.100	-0.242	0.334*	0.105	0.430**	-0.238	0.192	0.358*	-0.276	0.052	0.208	0.099
		N	41	41	41	41	41	41	41	41	41	41	41	41	41
		Correlation coefficent	-0.367	-0.469	-0.431	0.343	-0.134	0.344	-0.601	-0.018	0.078	-0.552	-0.492	-0.119	-0.177
		N	9	9	9	9	9	9	9	9	9	9	9	9	9
$PM_{2.5-10}$	IL-6	Correlation coefficent	-0.338*	0.300	0.122	0.206	-0.401*	-0.336*	-0.409**	-0.215	-0.274	0.078	-0.393*	-0.138	0.051
		N	40	40	40	40	40	40	40	40	40	40	40	40	40
		Correlation coefficent	-0.210	0.196	0.087	0.608	-0.279	-0.142	-0.306	-0.007	0.012	0.142	-0.248	-0.280	0.236
		N	9	9	9	9	9	9	9	9	9	9	9	9	9
$PM_{0.1-2.5}$	IL-6	Correlation coefficent	0.106	0.024	0.256	0.256	-0.031	-0.067	-0.045	0.15	-0.088	0.107	-0.115	-0.180	-0.158
		N	40	40	40	40	40	40	40	40	40	40	40	40	40
		Correlation coefficent	0.241	-0.156	0.041	0.825**	0.109	-0.085	-0.013	0.629	0.021	-0.066	-0.031	-0.316	-0.287
		N	9	9	9	9	9	9	9	9	9	9	9	9	9

Statistically significant correlations are shown as follow * $p<0.05$ ** $p<0.05$ ***$p<0.001$

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